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Research Article

Environmental DNA (eDNA) metabarcoding surveys show evidence of non-indigenous freshwater species invasion to new parts of Eastern Europe

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Abstract

Active environmental DNA (eDNA) surveillance through species-specific amplification has shown increased sensitivity in the detection of non-indigenous species (NIS) compared to traditional approaches. When many NIS are of interest, however, active surveillance decreases in cost- and time-efficiency. Passive surveillance through eDNA metabarcoding takes advantage of the complex DNA signal in environmental samples and facilitates the simultaneous detection of multiple species. While passive eDNA surveillance has previously detected NIS, comparative studies are essential to determine the ability of eDNA metabarcoding to accurately describe the range of invasion for multiple NIS versus alternative approaches. Here, we surveyed twelve sites, covering nine rivers across Belarus for NIS with three different techniques, i.e. an ichthyological, hydrobiological and eDNA survey, whereby DNA was extracted from 500 ml surface water samples and amplified with two 16S rDNA primer assays targeting the fish and macroinvertebrate biodiversity. Nine non-indigenous fish and ten non-indigenous benthic macroinvertebrates were detected by traditional surveys, while seven NIS eDNA signals were picked up, including four fish, one aquatic and two benthic macroinvertebrates. Passive eDNA surveillance extended the range of invasion further north for two invasive fish and identified a new NIS for Belarus, the freshwater jellyfish *Craspedacusta sowerbii*. False-negative detections for the eDNA survey might be attributed to: (i) preferential amplification of aquatic over benthic macroinvertebrates from surface water samples and (ii) an incomplete reference database. The evidence provided in this study recommends the implementation of both molecular-based and traditional approaches to maximise the probability of early detection of non-native organisms.

Key Words

aquatic, biodiversity assessment, Central European invasion corridor, fish, invasive species detection, macroinvertebrate, passive eDNA surveillance, traditional survey methods

Introduction

One of the main threats to native freshwater organisms is the establishment of and competition from non-indigenous species (NIS). In recent decades, this threat has intensified and accelerated through anthropogenic pressures, including climate change (Walther et al. 2009; Seebens et al. 2017). The introduction of NIS have the potential to transform local ecosystems through habitat transformation, community structure alteration and evolutionary process modification (Mooney and Cleland 2001; Gallardo et al. 2019; Linders et al. 2019), causing economic consequences, negative impacts on ecosystem services and human well-being (Gallardo et

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al. 2019). Early detection is, therefore, essential for NIS management (Simberloff et al. 2005; Trebitz et al. 2017).

Two factors have facilitated the invasion of Ponto-Caspian species into Belarusian rivers: (i) the secondary connection of isolated river basins (Bij de Vaate et al. 2002) and (ii) global climate change (Semenchenko and Rizevskiy 2017). Many rivers in Belarus find their origin across the national border in two historically isolated basins, the Baltic Sea (e.g. Daugava River, Neman River, Mukhavetc River) and Black Sea (e.g. Berezina River, Dnieper River, Pina River, Pripyat River, Sozh River) basins. To aid shipping transport within Belarus and throughout Europe, these two river basins have been secondarily connected via man-made canals (Bij de Vaate et al. 2002). The novel interconnectivity allowed Ponto-Caspian species to migrate north-westwards into Belarus (Karatayev et al. 2008). Global climate change is generating water temperatures that facilitate the reproductive success of NIS, enhancing their spread into Belarus from Kyiv (Ukraine), Kaunas (Lithuania) (Semenchenko and Rizevskiy 2017) and transboundary lakes and rivers.

Documenting the introduction and spread of NIS within Belarus commenced in the early 2000s (Semenchenko et al. 2009). The current freshwater NIS checklist of Belarus includes 24 species of benthic macroinvertebrates (Semenchenko et al. 2009; Semenchenko et al. 2016; Lipinskaya et al. 2018) and 14 species of fish (Semenchenko and Rizevskiy 2017), with the majority of detections occurring in the southern part of the country (Semenchenko et al. 2016; Semenchenko and Rizevskiy 2017; Lipinskaya et al. 2018). The implemented monitoring techniques included standard hydrobiological and ichthyological surveys, with taxonomic identification through morphological characteristics (Karatayev et al. 2008; Semenchenko et al. 2009; Mastitsky et al. 2010). While species identification is feasible and easily obtainable for certain taxonomic groups (e.g. vertebrates), taxonomic identification through morphological characteristics is challenging for the majority of phyla, further complicated by the presence of juvenile and damaged specimens during collection. DNA-based technologies have, therefore, been implemented in recent years and helped to identify new non-native amphipod (e.g. *Echinogammarus trichiatus* (Martynov, 1932) (Lipinskaya et al. 2018)) and fish species (e.g. *Proterorhinus semilunaris* (Heckel, 1837) (Golovenchik et al. 2020).

Environmental DNA (eDNA), defined as intra- and extracellular DNA obtained directly from environmental samples (e.g. soil, sediment, water) without an obvious source of biological material (Taberlet et al. 2012), has been used in the last decade for the detection of species (Ficetola et al. 2008; Goldberg et al. 2013) and the investigation of ecological communities (Thomsen et al. 2012; Brett et al. 2016), including the early detection of non-indigenous (Dougherty et al. 2016; Ardura and Planes 2017; Hinlo et al. 2017; Klymus et al. 2017) and elusive (Piaggio et al. 2014; Simpfendorfer et al. 2016) species. Initially, the early detection of NIS through aquatic eDNA focused on active surveillance using targeted species-specific assays to assess the presence of a single species, achieving a higher detection probability and sensitivity than traditional monitoring approaches (Ardura et al. 2015; Dougherty et al. 2016; Simpfendorfer et al. 2016). However, active surveillance decreases in cost- and time-efficiency when multiple NIS are of interest (Rojahn et al. 2021).

Therefore, a shift towards passive NIS surveillance has occurred more recently (Holman et al. 2019; van den Heuvel-Greve et al. 2021), with eDNA metabarcoding taking advantage of the complexity of the DNA signal contained within environmental samples and enabling the simultaneous detection of multiple species (Cristescu 2014). Although eDNA metabarcoding has outperformed traditional ichthyological survey techniques in multiple studies (Hänfling et al. 2016; Cilleros et al. 2019), active surveillance through targeted amplification has shown increased detection sensitivity for rare species compared to eDNA metabarcoding (Harper et al. 2018; Bylemans et al. 2019). While NIS have been detected by eDNA metabarcoding (Holman et al. 2019; van den Heuvel-Greve et al. 2021), comparisons of this approach to traditional survey techniques are needed to determine the capability of passive eDNA surveillance to accurately describe the invasion range of non-indigenous species.

In this study, the range of invasion for fish and macroinvertebrates in Belarusian rivers was determined by three survey techniques, i.e. an ichthyological, hydrobiological and eDNA metabarcoding survey. Our eDNA survey targeted two regions of the 16S rRNA gene for fish and crustacean detection. The number of NIS detected and the range of invasion of each NIS was compared between survey methods to determine the capability of eDNA metabarcoding to describe the invasion range of aquatic and benthic freshwater non-indigenous species in temperate riverine systems.

Materials and methods

Sampling sites

Twelve sites were sampled on nine water bodies across Belarus in May-June 2018 with three different monitoring methods to compare the detection efficiency of NIS between traditional survey techniques and eDNA metabarcoding (Suppl. material 1, Fig. 1). These sites represent a subset of areas where long-term monitoring is carried out by the standard hydrobiological and ichthyological surveys conducted in Belarus for NIS documentation (Semenchenko et al. 2013) and were chosen to increase the likelihood of NIS detection. For example, sampling sites N, ZD, NZ and PN are located close to the Belarusian border and a place of entry of NIS from Kyiv and Kaunas reservoirs. The sampling site on the Pina River (PP) is located close to a river port, a known entry point for aquatic NIS. The sampling site on the Mukhavetc River (B) represents a known entry point of NIS from Polish waters and sampling sites DM, BZ, S,

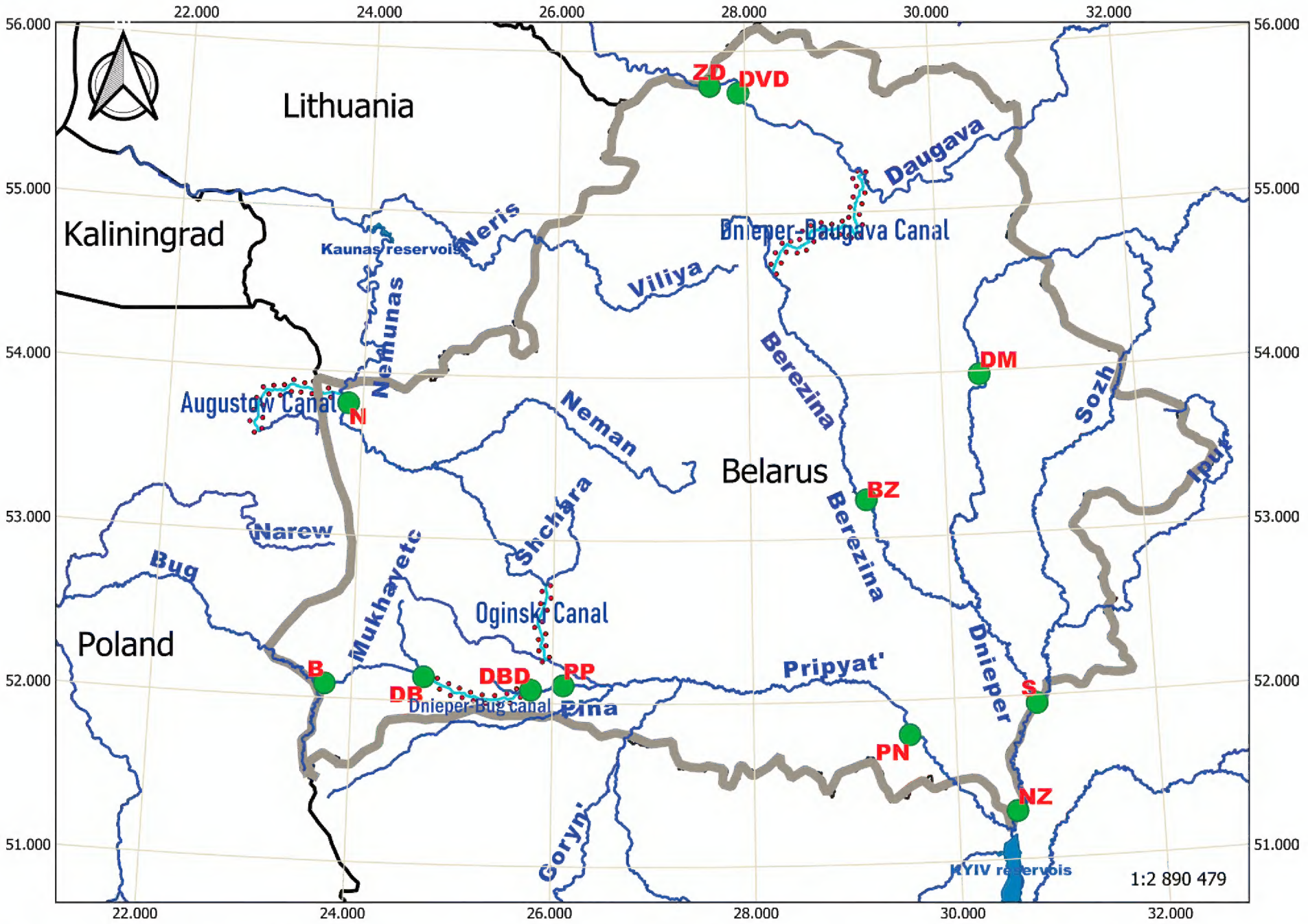

Figure 1. Map of Belarus displaying the twelve sampling sites. Sampling sites are indicated by green-coloured circles. Sampling site notation follows the abbreviations of Suppl. material 1.

DVD, DB and DBD were chosen to determine the range expansion of several established NIS in Belarus.

Sampling sites are characterised by different bottom structures and other environmental parameters (Suppl. material 1). Hydro-physical parameters (pH, conductivity, water temperature) were recorded by pH, EC/TDS and Temperature Meters HANNA HI 98311. The water pH varied during sampling from 6.8 to 8.5, conductivity from 210 µS to 396 µS and temperature varied from 19.5 °C to 23.4 °C.

Hydrobiological survey

Quantitative and qualitative benthic macroinvertebrate samples were taken by hand-net (ISO 7828; 25 cm × 25 cm frame; 500 µm mesh size) at each of the twelve sites. Two macroinvertebrate samples were obtained from the littoral zone of each site at a depth of 50 – 70 cm. For quantitative assessment, samples were collected by pushing the hand-net gently through the uppermost 2 – 5 cm of the substratum and dragging it for 3–5 m. For qualitative assessment, multiple smaller samples were collected from different habitats of the sample site to maximise the diversity of captured taxa. Samples were fixed in 96% ethanol and sorted in the laboratory. Specimens were identified to the lowest possible taxonomic level using identification keys, resulting in higher taxonomic ranks for certain groups, i.e. Hydrachnidia, Oligochaeta and Diptera. Moreover, juvenile and damaged specimens from the taxonomic groups of Mollusca, Ephemeroptera and Coleoptera were identified to the genera or family level.

Ichthyological survey

Two survey techniques were employed for the ichthyological survey dependent on the sampling site, seining (30 m length, 8 – 10 mm mesh size) and automatic folding umbrella type fishing net (80 cm × 80 cm frame; 5 mm mesh size). Ichthyological surveys were conducted in the littoral shallow part of the sampling sites. Species identification through morphological characteristics occurred on site. Native fish species were released back into their habitat upon identification, while non-indigenous species were collected for ichthyological and genetic purposes to the Laboratory of Ichthyology, Scientific and Practical Center for Bioresources, National Academy of Sciences of Belarus (Minsk, Belarus). Individual counts per species were used to infer abundance.

NIS barcoding

Three NIS (i.e. *Chelicorophium curvispinum* (G.O.Sars, 1895), *Obesogammarus obesus* (G.O.Sars, 1894) and *Neogobius fluviatilis* (Pallas, 1814)) without reference

sequences were barcoded for the 16S rDNA gene to expand the reference database and increase the potential for taxonomic identification from the eDNA survey. Specimens were taken from the morphologically identified collection (Ichthyological and Hydrobiological surveys). Genomic DNA was isolated from tissue samples using the Blood-Animal-Plant DNA Preparation Kit (Jena Bioscience, Germany), following the manufacturer's protocols with an overnight digestion step at 60 °C as a single modification. Barcodes were generated using the same primer sets as employed in the eDNA survey (Suppl. material 2).

PCR amplification was performed in 25 µl reactions, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, 1.5 units of Taq polymerase and 100 ng (1–3 µl) of template DNA. The thermocycling profile included an initial denaturation step of 95 °C for 5 minutes, 50 cycles of 95 °C for 30 seconds, 51–54 °C for 30 seconds for macroinvertebrates and fish, respectively and 72 °C for 45 seconds. A final extension step was performed at 72 °C for 10 minutes. PCR reactions were checked on 1% agarose gels stained with ethidium bromide. Size selection and clean-up were conducted using the PCR Purification Kit (Jena Bioscience, Germany) on successfully amplified samples. Bidirectional sequencing was conducted using dye-labelled terminators and PCR primers on the ABI 3130 (Applied Biosystems, Foster City, CA) genetic analyser with a BigDye Terminator v.3.1 cycle Sequencing Kit (Applied Biosystems, USA) at the Institute of Genetics and Cytology of the National Academy of Sciences of Belarus. Taxonomy and vouchers were deposited on the Barcode of Life Datasystem (BOLD) with the following accession numbers: *Ch. curvispinum* (TLAMP475S-17), *O. obesus* (TLAMP330S-17) and *N. fluviatilis* (689-fB).

Environmental DNA survey

Aquatic eDNA sampling was performed concurrent to the hydrobiological survey. Within each of the twelve sites, nine surface water samples were collected covering three habitats, with three biological replicate samples per habitat. Sampling occurred from 30 May until 10 June 2018. Environmental DNA filtration followed recommendations from (Spens et al. 2016). Briefly, pre-packed sterile 50 ml luer-lock syringes were used to push the sampled water through the Sterivex (Millipore, Merck KGaA, Darmstadt, Germany) column until clogging. The volume of water filtered through Sterivex columns ranged from 250 ml to 750 ml, depending on the turbidity of the water column. Mean volume of water per filter equalled 424.3 ± 124.8 ml (± SD), while mean volume of water per site equalled 3,641.67 ± 982.77 ml (± SD). Remaining water in the columns was removed by pushing air through the filter. Pre-packed sterile 5 ml luer-lock syringes were used to add 2 ml Longmire's Buffer (100 mM Tris, pH 8.0; 100 mM EDTA, pH 8.0; 10 mM NaCl; 0.5% sodium dodecyl sulphate; 0.2% sodium azide) to each column. Samples were stored at -20 °C until shipment on ice to the eDNA facility at the University of Otago, Dunedin, New Zealand, where samples were stored at -20 °C until further processing.

DNA extraction

Prior to laboratory work, all bench surfaces and equipment were sterilised by a 10 minute exposure to 10% bleach solution (Prince and Andrus 1992) and rinsed with ultrapure water. To test for contamination, negative filtration controls (500 ml ultrapure water), negative extraction controls (500 µl ultrapure water) and negative PCR controls (2 µl ultrapure water) were added and processed alongside the samples.

Sample processing followed the recommendations in Spens et al. (2016) with slight modifications. Briefly, DNA was extracted solely from the Longmire's Buffer, since DNA extracts from filter capsules did not show amplification success during initial testing, most likely due to the lysis and leaching of DNA into the Longmire's Buffer (Williams et al. 2016; David et al. 2021). Caps were taken off Sterivex columns and buffer was transferred to a 2 ml Eppendorf LoBind tube using a pre-packed sterile 5 ml luer-lock syringe. Samples were spun at 6000× g for 45 minutes, after which the supernatant was discarded. 180 µl ATL and 20 µl proteinase K were added to the pellet. Samples were briefly vortexed and incubated at 56 °C overnight in a spinning rotor. Following 15 seconds of vortexing, equal volumes of buffer AL and 100% ethanol were added to the sample. After mixing, the standard protocol of the Qiagen DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) was followed. DNA extracts were stored at -20 °C until further processing.

Library preparation

Library preparation followed the protocol described in (Jeunen et al. 2018). Briefly, two metabarcoding assays targeting two fragments of the 16S rDNA gene region were used to amplify DNA from fish and crustaceans (Suppl. material 2). Prior to library preparation, the presence of inhibitors was tested for and low-template samples were identified by a dilution series (neat, 1/10, 1/20) and qPCR analysis (Murray et al. 2015). Amplification was carried out in triplicate in 25 µl reactions to account for variation in amplification results in low-template samples. qPCR mastermix consisted of 1× SensiFAST SYBR Lo-ROX Mix (Bioline, London, UK), 0.4 µmol/l of each primer (Integrated DNA Technologies, Australia), 2 µl of template DNA and ultrapure water as required. The thermal cycling profile included an initial denaturation step at 95 °C for 10 minutes; then 50 cycles of 30 seconds at 95 °C, 30 seconds at 51–54 °C (see annealing temperatures in Suppl. material 2), 45 seconds at 72 °C; and a final extension of 10 minutes at 72 °C.

A one-step amplification protocol using fusion primers was employed for library building (Berry et al. 2017).

Fusion primers contained an Illumina adapter, a modified Illumina sequencing primer (absent in the reverse fusion primer), a barcode tag (6–8 bp in length) and the template specific primer (Murray et al. 2015). Each sample was amplified in duplicate to counteract effects of PCR stochasticity (Leray and Knowlton 2015; Alberdi et al. 2018) and assigned a unique barcode combination. qPCR conditions followed the protocol described for the inhibition test. Post qPCR, sample duplicates were pooled to reduce stochastic effects from PCR amplification. Samples were then pooled, based on end-point qPCR fluorescence and Ct-value in mini pools. Size selection and qPCR clean-up followed the AMPure XP (Beckman Coulter, US) standard protocol. Molarity of mini pools was measured on Agilent 2100 Bioanalyzer (Agilent, The Netherlands) and pooling occurred equimolarly to produce a single DNA library. Final concentration of the library was assessed via Qubit. Sequencing was performed by Otago Genomics on Illumina MiSeq (300 cycle, single-end V2 kit), following the manufacturer's protocols, with 10% of PhiX to minimise issues associated with low-complexity libraries.

Bioinformatic and statistical analyses

The bioinformatic analysis for both assays followed an in-house bioinformatic pipeline using FastQC v.0.11.5 (Bioinformatics 2011), Geneious Prime v.11.0.3+7 (Kearse et al. 2012), VSEARCH v.2.13.3 (Rognes et al. 2016) and OBITools v.1.2.11 (Boyer et al. 2016). Raw fastq files were checked for quality using FastQC. Reads were separated by barcode and assigned to samples using the 'separate reads by barcode' function in Geneious Prime, allowing for a single mismatch. All barcodes had a minimum three basepair mismatch distance from each other. Primer sequences were removed, allowing for a single mismatch, using the 'annotate new trimmed regions' function in Geneious Prime. The remaining reads were exported in fastq format and subsequently filtered, based on total expected errors "--fastq_maxee 0.1", minimum length "--fastq_minlen 100", maximum length "--fastq_maxlen 230" and ambiguous bases "--fastq_maxns 0", using the '--fastq_filter' function in VSEARCH. Successful quality filtering was checked by FastQC report. The remaining sequences were dereplicated into unique sequences using the '--derep_fulllength' function and unique sequences with an abundance lower than 50 were removed. Unique sequences were clustered at 97% using the '--cluster_size' function, followed by the removal of chimeric sequences with the function '--uchime3_denovo'. Finally, an OTU table was generated at 97% threshold using the '--usearch_global' function.

All OTUs were assigned a taxonomy using the 'ecotag' function in OBITools (Boyer et al. 2016), based on a global alignment algorithm (Needleman and Wunsch 1970). To assign taxonomy using the 'ecotag' function, a custom reference database was generated for both metabarcoding assays by an *in silico* PCR using the 'ecoPCR' function on the EMBL dataset (downloaded on 13 May 2020; Suppl. material 3). The reference databases were generated using the example code provided in the OBITools tutorial (https://pythonhosted.org/OBITools/wolves.html; section 'Taxonomic assignment of sequences'). The custom reference databases were supplemented with the newly-barcoded NIS sequences (see Methods section *NIS barcoding*). Further filtering was conducted on the taxonomic assignment table prior to statistical analysis. All OTUs, failing to obtain a taxonomic assignment, were discarded from the dataset, as well as identification of unspecific co-amplified taxonomic groups not targeted by the traditional surveys, a positive detection in a sample represented by a single sequence and OTUs with positive detections in negative control samples. Finally, identical taxonomic assignments were summed per sample and replicates per site were summed to obtain a single taxonomic list per sampling site.

We checked the reference database for the presence of all NIS (both fish and benthic macroinvertebrates), detected by the traditional survey methods. In case of missing reference sequences, we attempted to barcode voucher specimens if tissue samples were available (see Methods section *NIS barcoding*). Furthermore, the 'ecoPCR' function in OBITools provides information about mismatches in the primer-binding region, an estimate for amplification efficiency. Mismatches in the primer-binding region were visualised in Fig. 2 for all NIS. Rarefaction curves were generated to assess sequencing coverage using the 'rarecurve' function from the 'vegan v.2.4-1.' package in R v.3.3.2 (R; http://www.R-project.org). Bioinformatic and R scripts can be found in Suppl. material 4.

Results

Biodiversity detection

A total of 43 fish species were identified across the twelve sampling sites with our ichthyological survey, representing twelve families and eight orders, including Cypriniformes, Perciformes, Syngnathiformes, Osmeriformes, Gadiformes, Siluriformes, Clupeiformes and Salmoniformes (Suppl. material 5).

Our hydrobiological survey identified a total of 133 macroinvertebrate taxa across all twelve sampling sites, covering 66 families and four phyla, i.e. Cnidaria, Mollusca, Annelida and Arthropoda (Suppl. material 6). Taxonomic assignment through morphological characteristics allowed us to identify 97 taxa to species level, while 19 taxa were identified to genus and 17 taxa to family level.

Filtering and quality control returned 5,661,054 reads, with 3,845,772 and 1,815,282 reads for the fish (16S) and crustacean (16S) metabarcoding assays, respectively. Overall, eDNA samples achieved good sequencing coverage, based on rarefaction curves reaching saturation for all samples (Suppl. material 7), with a mean number of reads per habitat ± s.d.: fish (16S): 113,662 ±137,606; crustacean (16S): 51,932 ± 35,319. Amplification

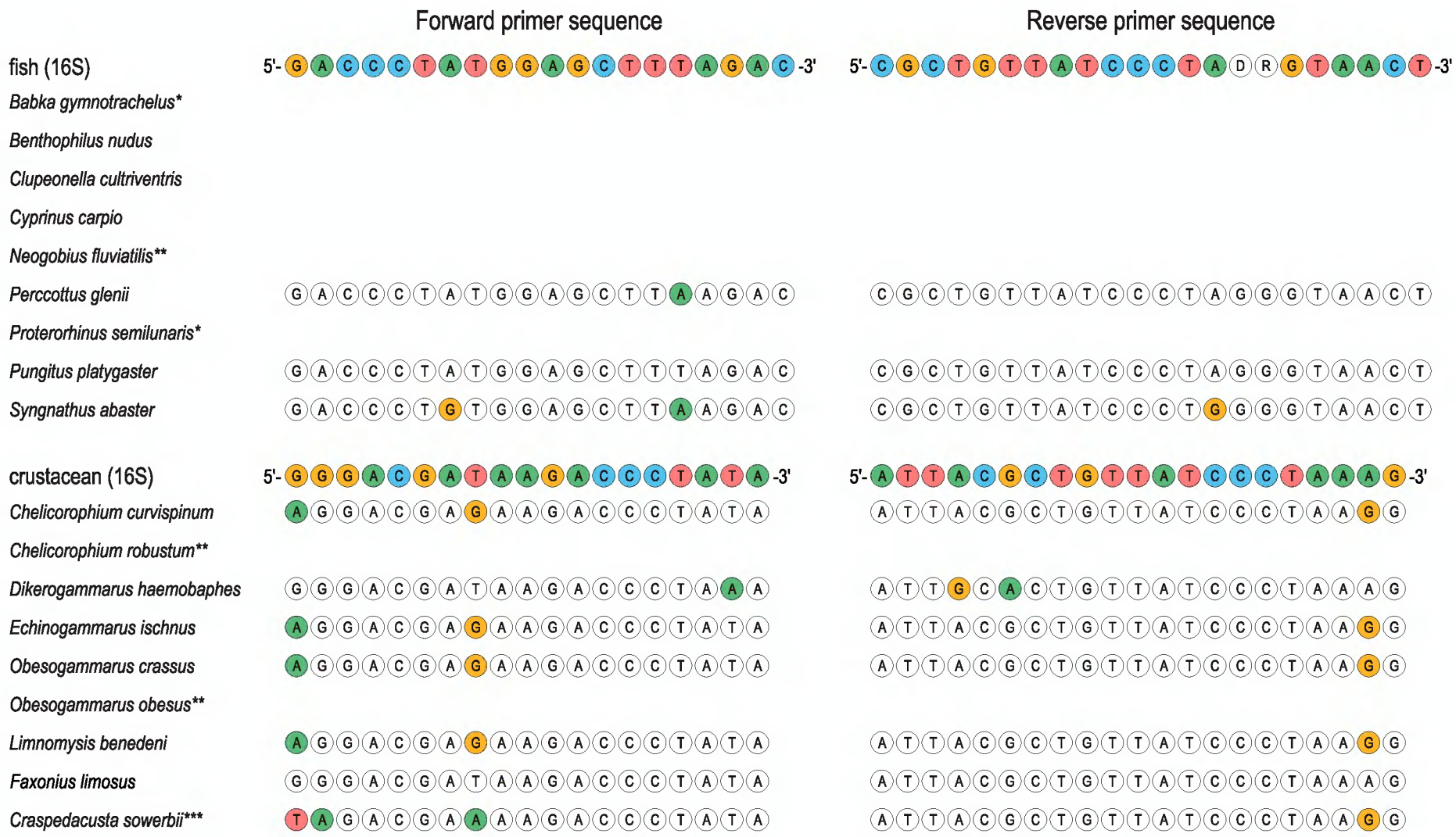

Figure 2. *In silico* PCR analysis identifying the completeness of the reference database and mismatches in the forward and reverse primer binding site for the fish (16S) and crustacean (16S) assay. Mismatches in the primer binding sites are indicated by coloured circles. * denotes the presence of a reference sequence without primer-binding regions, ** denotes newly-barcoded species and *** denotes species only detected by the eDNA survey. Species with missing primer information are a result of incomplete reference information in the database.

difficulties, resulting in low coverage, were encountered in sample 3-N for the fish (16S) assay. This sample was removed from the final dataset, prior to analysis. The number of sequences assigned to negative controls ranged between 0 and 33 for the fish (16S) metabarcoding assay and only a single sequence was assigned to one negative control for the crustacean (16S) metabarcoding assay. OTUs with a positive detection in a negative control were removed from the dataset prior to statistical analysis. A total of 31 and 246 OTUs were recovered for the fish (16S) and crustacean (16S) metabarcoding assays, respectively. Further stringent quality control post taxonomic assignment reduced the number of total detections to 15 and 75, covering 8 and 36 families for the fish (16S) and crustacean (16S) metabarcoding assays, respectively (Suppl. material 8).

Non-indigenous species detection

Across all survey methods, we were able to detect twenty non-indigenous species, including nine fish and eleven macroinvertebrates. All non-indigenous species were previously recorded in Belarus, except for a freshwater jellyfish (*Craspedacusta sowerbii* Lankester, 1880), which was only detected by our eDNA metabarcoding survey. For non-indigenous fish, our ichthyological survey detected all nine species, including racer goby (*Babka gymnotrachelus* (Kessler, 1857)), monkey goby (*N. fluviatilis*), western tubenose goby (*Pr. semilunaris*), Chinese sleeper (*Perccottus glenii* Dybowski, 1877), black-striped pipefish (*Syngnathus abaster* Risso, 1827), southern nine spine stickleback (*Pungitius platygaster* (Kessler, 1859)), Black Sea tadpole-goby (*Benthophilus nudus* Berg, 1898), common carp (*Cyprinus carpio carpio* Linnaeus, 1758) and Black Sea sprat (*Clupeonella cultriventris* (Nordmann, 1840)). Our eDNA metabarcoding survey detected four non-indigenous fish, including racer goby (*B. gymnotrachelus*), western tubenose goby (*Pr. semilunaris*), Chinese sleeper (*P. glenii*) and monkey goby (*N. fluviatilis*). The number of fish NIS detected per sampling site was equally distributed between eDNA-only detection (31.2%), shared detection (35.9%) and ichthyological-only detection (32.8%; Table 1). Furthermore, species occurrence was also equally distributed between eDNA-only detection (27.8%), shared detection (36.1%) and ichthyological-only detection (36.1%; Table 2).

Ten out of 24 established non-indigenous macroinvertebrates were detected by the 2018 hydrobiological survey. These include one mysid (*Limnomysis benedeni* Czerniavsky, 1882), six amphipods (*Chelicorophium robustum* (G.O.Sars, 1895), *Ch. Curvispinum*, *Dikerogammarus haemobaphes* (Eichwald, 1841), *Echinogammarus ischnus* (Stebbing, 1899), *Obesogammarus crassus* (G.O.Sars, 1894) and *O. obesus*), one decapod (*Faxonius limosus* (Rafinesque, 1817)) and two invasive alien molluscs (*Lithoglyphus naticoides* (C.Pfeiffer, 1828) and *Dreissena polymorpha* (Pallas, 1771)). Both alien molluscs were excluded in the comparative analysis, as the

Table 1. Number of species detected per sampling site with the ichthyological, hydrobiological and eDNA metabarcoding surveys. Data for the eDNA metabarcoding survey are split up into the two different taxonomic groups, with the fish (16S) assay representing the fish NIS detections and the crustacean (16S) assay representing the macroinvertebrate NIS detections. Numbers in brackets indicate values when disregarding NIS detections without a reference barcode. Sampling site notation follows the abbreviations of Suppl. material 1.

Taxonomic group	Detection	Sampling sites												Total
		B	N	DB	DBD	PP	ZD	DVD	DM	BZ	PN	NZ	S	
fish	eDNA	1	2	1	2	1	1 (1)	0 (0)	0	0	0	0 (0)	2	9 (9)
	shared	2	0	1	0	3	0 (0)	0 (0)	2	1	3	1 (1)	0	12 (12)
	ichthyological	0	0	1	1	0	1 (0)	1 (0)	1	0	1	6 (4)	1	13 (9)
	sum	3	2	3	3	4	2 (1)	1 (0)	3	1	4	7 (5)	3	36 (32)
	eDNA (%)	33.3	100	33.3	66.7	25	50 (100)	0 (0)	0	0	0	0 (0)	66.7	31.2 (38.6)
	shared (%)	66.7	0	33.3	0	75	0 (0)	0 (0)	66.7	100	75	14.3 (20.0)	0	35.9 (39.7)
	ichthyological (%)	0	0	33.3	33.3	0	50 (0)	100 (0)	33.3	0	25	85.7 (80.0)	33.3	32.8 (21.7)
macroinvertebrates	eDNA	0	1	0	0	1	0	0	1	2	1	0	0	6
	shared	2	0	0	0	0	0	0	0	0	0	0	0	2
	hydrobiological	1	3	0	1	1	0	0	1	0	6	6	2	21
	sum	3	4	0	1	2	0	0	2	2	7	6	2	29
	eDNA (%)	0	25	0	0	50	0	0	50	100	14.3	0	0	26.6
	shared (%)	66.7	0	0	0	0	0	0	0	0	0	0	0	7.4
	hydrobiological (%)	33.3	75	0	100	50	0	0	50	0	85.7	100	100	66.0

phylum Mollusca is not amplifiable by the primer assays used in our eDNA survey. Our eDNA metabarcoding survey only detected three non-indigenous macroinvertebrate species, two of which were also detected by our hydrobiological survey, i.e. the spinycheek crayfish (*F. limosus*) and *D. haemobaphes*. Our eDNA survey detected one additional NIS, a freshwater jellyfish (*Cr. sowerbii*), not detected by the hydrobiological survey. The number of macroinvertebrate NIS detected per sampling site was mostly represented by the hydrobiological survey (66.0%), while 26.6% accounted for eDNA-only detections and overlap between survey methods was limited to two species at a single site, accounting for 7.4% of NIS detections (Table 1). Furthermore, species occurrence was also mostly represented by the hydrobiological survey (72.4%), while 20.7% of occurrences were represented by eDNA-only detections and 6.9% of species occurrences were shared between survey methods (Table 2).

Table 2. Number of occurrences each NIS was detected by the ichthyological, hydrobiological and eDNA metabarcoding surveys. Data for the eDNA metabarcoding survey are split up into the two different taxonomic groups, with the fish (16S) assay representing the fish NIS detections and the crustacean (16S) assay representing the macroinvertebrate NIS detections. * denotes NIS without a reference barcode sequence. ** denotes species only detected by the eDNA metabarcoding survey.

Taxonomic group	Species	eDNA	shared	Traditional
fish	*Babka gymnotrachelus*	4	6	0
	**Benthophilus nudus*	0	0	1
	**Clupeonella cultriventris*	0	0	1
	**Cyprinus carpio*	0	0	2
	Neogobius fluviatilis	0	5	4
	Perccottus glenii	5	0	1
	Proterorhinus semilunaris	1	2	2
	Pungitius platygaster	0	0	1
	Syngnathus abaster	0	0	1
macroinvertebrates	*Chelicorophium curvispinum*	0	0	4
	Chelicorophium robustum	0	0	2
	Dikerogammarus haemobaphes	1	1	6
	Echinogammarus ischnus	0	0	2
	Obesogammarus crassus	0	0	3
	Obesogammarus obesus	0	0	1
	Limnomysis benedeni	0	0	2
	Faxonius limosus	0	1	1
	***Craspedacusta sowerbii*	5	0	0

Reference database analysis

Overall, eleven of the seventeen NIS detected by both traditional monitoring methods, including five out of nine fish and six out of eight macroinvertebrates, have a reference barcode in molecular databases for the 16S target region of our eDNA metabarcoding assays (Fig. 2). The *in silico* PCR used to construct the reference database requires the presence of primer-binding sites, which are frequently removed prior to sequence depositing in the EMBL database when the same assay is used for barcoding. Therefore, two of the five non-indigenous fish were not picked up by the *in silico* PCR analysis, even though a reference for the amplicon is publicly available on the EMBL database. For this project, one non-indigenous fish (*N. fluviatilis*, BOLD accession number 689-fB) and two non-indigenous macroinvertebrates (*Ch. curvispinum*, BOLD accession number TLAMP475S-17; *O. obesus*, BOLD accession number TLAMP330S-17) were barcoded. At the time of analysis, *B. nudus*, *Cl. cultriventris* and *C. carpio carpio* do not have a reference barcode for the target region of the fish (16S) assay and, hence, cannot be identified, at least to species-level, by our eDNA metabarcoding survey.

Two of the nine NIS picked up in our *in silico* PCR did not display mismatches in the primer-binding sites, including one fish (*P. platygaster*) and one invertebrate (*F. limosus*). One fish (*P. glenii*) displayed a single mismatch in the primer-binding sites, while the remaining NIS displayed three mismatches. Mismatches in the

forward primer were found in the 5' end, while mismatches in the reverse primer were found in the 3' end for crustacean NIS, potentially influencing amplification efficiency for this taxonomic group.

Locating the range of invasion – fish NIS

Our ichthyological survey detected seven of the nine NIS on the Dnieper River (site NZ) near the southern border with Ukraine, the entry point of invasion (Fig. 3). Four NIS (*B. gymnotrachelus*, *Cl. cultriventris*, *P. platygaster* and *S. abaster*) were only found at site NZ in low abundance. The most widely distributed NIS according to our ichthyological survey was *N. fluviatilis*, followed by *B. gymnotrachelus* and *Pr. semilunaris*. All three species were detected at multiple sites throughout the southern region of Belarus on the Dnieper and Pripyat Rivers. Two NIS were not detected at site NZ. *P. glenii* was only detected at site PN, while *C. carpio* was detected in the two most northern sites, i.e. site ZD and site DVD. Non-indigenous fish were found at eleven sites, excluding one site on the Neman River (site N). The highest number of NIS fish (seven species) was detected on the Dnieper River (site NZ), followed by five NIS fish on the Pina River (site PP). Highest abundance of NIS fish (13.59%) was detected on the Dnieper River (site NZ), followed by 11.74% and 11.68% on the Pripyat River (site PN) and Dnieper-Bug canal (site DBD), respectively.

Our fish eDNA metabarcoding survey detected four of the six non-indigenous fish species with a reference barcode, while failing to detect two fish NIS, i.e. *P. platygaster* and *S. abaster*, both detected in low abundance at a single site by our ichthyological survey (Fig. 3). The most widely distributed NIS according to our eDNA survey was *B. gymnotrachelus*, followed by *N. fluviatilis*, *P. glenii* and *Pr. semilunaris*. All NIS were detected in the southern region of Belarus and were detected further north compared to our ichthyological survey (Fig. 3). Non-indigenous fish were found at eleven sites, excluding one site on the Daugava River

Figure 3. Site-specific non-indigenous fish detection for the ichthyological and eDNA surveys. Positive detection is indicated by coloured cells, with a positive detection for the eDNA survey in orange and positive detection for the ichthyological survey in blue. * denotes NIS without a reference barcode sequence. Sampling site notation follows the abbreviations of Suppl. material 1.

(site DVD). All non-indigenous fish were detected on the Pina River (site PP), while three NIS were detected on the Mukhavetc River (site B) and Pripyat River (site PN). Based on relative number of reads, highest abundance of NIS fish (40.25%) was detected on the Dnieper River (site NZ), followed by 27.54%, 21.85% and 20.56% on the Dnieper River (site DM), Pina River (site PP) and Dnieper-Bug canal (site DBD), respectively.

Locating the range of invasion – invertebrate NIS

Non-indigenous macroinvertebrates were recorded at all twelve sites. The highest number of NIS (seven species) was detected on the Pripyat River (site PN), followed by six NIS on the Dnieper River (site NZ) and five NIS on the Mukhavetc River (site B). Highest abundance of NIS (405 individuals) was detected on the Pripyat River (site PN), followed by 81 and 80 individuals on the Dnieper River (site NZ) and Sozh River (site S), respectively (Suppl. material 6). According to the hydrobiological survey, the gravel snail (*L. naticoides*) is most widely distributed with a positive detection at ten sites, followed by *D. haemobaphes* with a positive detection at seven sites (Fig. 4). The highest abundant NIS were *O. crassus* and *L. naticoides*, with 331 and 174 detections, respectively. The least widely distributed NIS with a detection at a single site (site PN) was *O. obesus*, followed by *F. limosus*, *L. benedeni*, *E. ischnus* and *Ch. robustum*, which were detected at two sites. The least abundant NIS were *F. limosus*, *O. obesus* and *Ch. robustum*, with two, two and three detections, respectively (Suppl. material 6).

Our eDNA metabarcoding survey detected only two of the eight non-indigenous macroinvertebrates with a reference barcode, i.e. *D. haemobaphes* and *F. limosus* (Fig. 4). For both invasive species, the eDNA survey obtained a positive detection in a reduced number of sites compared to the hydrobiological survey. However, the eDNA survey detected *D. haemobaphes* at one additional site on the Berezina River (site BZ). The eDNA survey detected an additional NIS, a freshwater jellyfish

Figure 4. Site-specific non-indigenous macroinvertebrate detection for the hydrobiological and eDNA surveys. Positive detection is indicated by coloured cells, with a positive detection for the eDNA survey in orange and positive detection for the hydrobiological survey in blue. * denotes NIS detected solely by the eDNA survey. Sampling site notation follows the abbreviations of Suppl. material 1

(*Cr. sowerbii*), not detected by the hydrobiological survey. *Craspedacusta sowerbii* is a known European invader, previously not yet reported in Belarussian rivers and lakes, but was found in several artificial water bodies in the Pripyat and Mukhavetc River Basins by local people. According to the eDNA survey, the freshwater jellyfish (*Cr. sowerbii*) was most widely distributed with a positive eDNA signal at five sites, while *D. haemobaphes* was only detected in two out of seven sites compared to the hydrobiological survey and the spinycheek crayfish (*F. limosus*) was only detected at a single site on the Mukhavetc River (site B) compared to two sites in the hydrobiological survey (Fig. 4).

Discussion

In this study, we evaluated eDNA metabarcoding as an alternative survey method for the simultaneous detection of non-indigenous species in riverine systems. Our results provide compelling evidence that eDNA metabarcoding on DNA extracted from surface water samples can be implemented for aquatic NIS monitoring in freshwater environments to explore the range of invasion (Hinlo et al. 2017; Holman et al. 2019; Suarez-Menendez et al. 2020; Chen et al. 2021; van den Heuvel-Greve et al. 2021). However, our eDNA metabarcoding survey from surface water samples failed to reliably detect benthic macroinvertebrates. The complexity of the DNA signal from environmental samples, furthermore, enables the detection of unexpected NIS (e.g. the freshwater jellyfish *Cr. sowerbii*) that are not targeted through established traditional monitoring methods, such as the ichthyological and hydrobiological surveys currently conducted in Belarus.

By detecting an increased range of invasion for two non-indigenous fish species, we document the potential of eDNA metabarcoding from low-volume surface-water samples to detect aquatic NIS at an early stage of invasion. For example, the eDNA survey detected *B. gymnotrachelus* in the Neman and Daugava Rivers, which represents a potential range extension of this species into this part of Belarus (Semenchenko et al. 2011). Additionally, our eDNA metabarcoding survey detected the Chinese sleeper (*Perccottus glenii*) further west along the Pripyat River compared to the ichthyological survey. These results indicate that incorporating eDNA metabarcoding surveys into established conservation programmes has the potential to increase the chance for successful eradication of aquatic NIS, as eDNA facilitates early detection (Reaser et al. 2020). Without physical evidence of sighted specimens, however, we recommend increased monitoring at these sampling sites to validate the eDNA metabarcoding results. Once validated, eDNA metabarcoding surveys could be used as a guide for increased monitoring efforts at specific locations.

While our results provide evidence for an increased sensitivity of eDNA over traditional monitoring approaches, in agreement with previously published research (Ardura et al. 2015; Dougherty et al. 2016; Simpfendorfer et al. 2016), we also observed false-negative fish NIS detections for our eDNA metabarcoding survey. False-negative detections could partially be attributed to missing barcode sequences. Three NIS missed by our eDNA survey currently do not have a reference barcode available on public databases for the 16S rDNA gene targeted by the fish (16S) assay (Fig. 2). With eDNA metabarcoding relying on species-identification through reference barcodes, these three species might have been picked up by eDNA, but unable to be resolved to species level (Hestetun et al. 2020). Continuous barcoding efforts of multiple genetic markers or complete mitogenomes will be essential to aid taxonomy assignment for eDNA metabarcoding surveys in the future (Collins et al. 2019).

False-negative fish NIS detections might also be explained by low amplification efficiency, which could reduce the probability of detecting rare eDNA molecules. The *in silico* PCR analysis revealed multiple mismatches in the forward and reverse primer-binding sites for the majority of target NIS (Fig. 2), which may reduce amplification efficiency (Stadhouders et al. 2010). Future assay optimisation could decrease primer mismatches and improve amplification efficiency and, hence, the detection probability for target non-indigenous species. Furthermore, inclusion of blocking primers to exclude DNA signals originating from the host organism in dietary studies (Robeson II et al. 2018) or highly abundant species in environmental monitoring has shown to increase the detection probability for rare species and reduce the minimum required sampling effort (Wilcox et al. 2014; Rojahn et al. 2021). As NIS might only contribute a small proportion of the total biomass during early settlement, the use of blocking primers of highly abundant eDNA signals might increase the detection efficiency for rare organisms. The amplification efficiency might have been further reduced by the use of fusion primers during library preparation, which exhibit lower amplification efficiency compared to a two-step protocol (Murray et al. 2015; Schnell et al. 2015; Bohmann et al. 2022). While a comparative study between library protocols revealed no significant difference in species detection for mock communities (Zizka et al. 2019), additional research investigating the impact of library preparation protocols on eDNA metabarcoding is required.

Finally, false-negative fish NIS detections could also be a consequence of our experimental design. For eDNA capture, we opted to employ the frequently used Sterivex filters with a pore size of 0.22 µm (Spens et al. 2016). However, the high turbidity at our sampling sites limited the volume processed until the filter clogged. Since processing larger volumes has been shown to reduce the risk of false-negative detections (Li et al. 2018; Cantera et al. 2019), larger pore-sized filters might, therefore, be a more suitable alternative for turbid environments and reduce false-negative NIS detections. The long transport time of samples from Belarus to New Zealand prior to eDNA extraction could also have attributed to false-negative fish NIS detections. While filter storage in

Longmire's Buffer has been shown to effectively preserve eDNA over short periods of time (Renshaw et al. 2015), immediate DNA extraction is preferred (Kumar et al. 2020). If limited, but continuous, eDNA degradation occurred during transport, this could have resulted in a failure to detect low-abundant fish, such as the black-striped pipefish (*S. abaster*) and the southern nine spine stickleback (*P. platygaster*) in the southernmost site (NZ). Another explanation for the false-negative detections might be related to the time difference when the eDNA and ichthyological surveys were conducted. High spatial and temporal resolutions have been reported for aquatic eDNA (Beentjes et al. 2019; Brys et al. 2020), contributing to the high accuracy in species detection. However, high spatial and temporal resolutions could also result in a need for increased sampling effort to reliably detect migrating or non-established species.

Besides the range extension of two non-indigenous fish species, the eDNA survey detected one additional aquatic NIS with the crustacean (16S) assay, i.e. the freshwater jellyfish *Cr. sowerbii*. While both traditional monitoring methods employed in Belarus are field standards, they target specific taxonomic groups that do not cover invertebrate organisms residing in the water column. Environmental DNA metabarcoding, on the other hand, takes advantage of the complexity of the DNA signal from environmental samples, facilitating the detection of unexpected NIS, providing a reference barcode is available. *Cr. sowerbii* natively inhabits freshwater bodies of Eastern Asia (Jankowski et al. 2008) and was first recorded in Europe (United Kingdom) in 1880 (Boothroyd et al. 2002) and 1901 in mainland Europe (Lytle 1960). While *Cr. sowerbii* has been recorded in neighbouring countries, such as Ukraine and Poland (Arbačiauskas and Lesutienė 2005; Didžiulis and Zurek 2013), this is the first record of the freshwater invasive jellyfish in Belarus. The role of freshwater jellyfish in food webs, as well as their impact on local aquatic communities still remains insufficiently studied (Dumont 1994). While the direct impact for Belarusian riverine communities might be restricted to the predation of fish eggs (Dumont 1994), *Craspedacusta sowerbii* might secondarily enhance the spread of the non-indigenous spinycheek crayfish (*F. limosus*), which actively predates on this freshwater jellyfish under laboratory conditions (Dodson and Cooper 1983). The presence of *Cr. sowerbii* might, therefore, increase the available food source of this alien crayfish. Two months after our eDNA survey, this species was detected through traditional monitoring and confirmed in Belarusian waters. Further studies are required to determine the impact of *Cr. sowerbii* on the native riverine communities.

Our eDNA metabarcoding survey failed to reliably detect non-indigenous, benthic macroinvertebrates. Given the majority of detected taxa for the crustacean (16S) assay consisted of aquatic and aquatic-associated invertebrates (e.g. copepods and dragonflies; Suppl. material 8), our results potentially indicate the need to sample a different substrate to reliably detect benthic macroinvertebrates. Previous studies have reported different eDNA signals obtained from various substrates originating from the residing community (Turner et al. 2015; Koziol et al. 2019). Furthermore, different eDNA signals have also been obtained from different depths in the water column in stratified conditions (Jeunen et al. 2019; Littlefair et al. 2020). Additionally, the macroinvertebrate eDNA signal retrieved from the water column in riverine systems has been shown to differ from the detected diversity from benthic bulk samples, indicating aqueous eDNA might not be effective at detecting benthic taxa (Gleason et al. 2020). The results obtained in our study corroborate these findings, with the failure to reliably detect non-indigenous, benthic macroinvertebrates potentially further exacerbated by the aforementioned factors influencing fish NIS detection. Though the inclusion of sediment sampling alongside water sampling in future eDNA metabarcoding surveys in this region is required to validate this hypothesis. Although eDNA metabarcoding has the potential to aid monitoring efforts in the early detection of NIS, data obtained from a single substrate might be insufficient when targeting taxonomic groups inhabiting various substrates (e.g. water column vs. sediment). A more substantial sampling strategy incorporating multiple substrates could, therefore, be recommended when targeting various taxonomic groups inhabiting different substrates.

Conclusions

With this comparative experiment, we provide evidence for the potential of eDNA metabarcoding to record the invasion range of multiple non-indigenous aquatic species in an accurate, cost-effective and time-efficient manner. In agreement with previously published research, we show that aquatic eDNA metabarcoding has the potential to aid monitoring efforts in the early detection of aquatic NIS and guide future monitoring efforts to specific locations. Furthermore, by taking advantage of the complex DNA signal contained within environmental samples, eDNA metabarcoding increases the chance to detect unexpected NIS. However, surface water eDNA signals failed to reliably detect benthic macroinvertebrates, thereby showing that a sampling strategy incorporating multiple substrates might be required when NIS inhabiting different niches are targeted. We, therefore, recommend the implementation of eDNA metabarcoding surveys alongside traditional approaches to increase the probability of early NIS detection and, hence, facilitate successful eradication efforts and minimise ecological impacts.

Data availability statement

The demultiplexed sequencing data (separate fastq files after Geneious Prime processing) has been uploaded to SRA (Sequence Read Archive) under submission number SUB11515362 and BioProject ID PRJNA841690.

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Supplementary material 1
Sampling sites and their description
Author: Gert-Jan Jeunen
Data type: Table including sampling site metadata
Explanation note: Sampling sites and their description.
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Link: https://doi.org/10.3897/mbmg.6.68575.suppl1

Supplementary material 2
Metabarcoding qPCR assays and the respective primer sets used for biodiversity detection
Author: Gert-Jan Jeunen
Data type: eDNA primer information
Explanation note: Metabarcoding qPCR assays and the respective primer sets used for biodiversity detection.
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Link: https://doi.org/10.3897/mbmg.6.68575.suppl2

Supplementary material 3
Reference databases generated by ecoPCR and used by ecotag for taxonomy assignment of OTUs for fish and crustacean eDNA results
Author: Gert-Jan Jeunen
Data type: Reference database used for eDNA taxonomy assignment.
Explanation note: Reference databases generated by ecoPCR and used by ecotage for taxonomy assignment of OTUs for fish and crustacean eDNA results.
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Link: https://doi.org/10.3897/mbmg.6.68575.suppl3

Supplementary material 4
Bioinformatic and statistical scripts used to process eDNA data
Author: Gert-Jan Jeunen
Data type: Bioinformatic and statistical scripts.
Explanation note: Bioinformatic and statistical scripts used to process eDNA data.
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Link: https://doi.org/10.3897/mbmg.6.68575.suppl4

Supplementary material 5
Relative fish abundances as observed by the ichthyological survey
Author: Tatsiana Lipinskaya
Data type: Survey data
Explanation note: Relative fish abundances as observed by the ichthyological survey. Scientific and common names are given in the respective columns. Non-indigenous species are identified by "Yes" in the "Invasive" column. Values indicate the relative abundance at a given sampling site, while "+" indicates a positive detection at sampling sites "ZD" and "DVD". Sampling site notation follows the abbreviations of Table 1.
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Link: https://doi.org/10.3897/mbmg.6.68575.suppl5

Supplementary material 6
Macro-invertebrate abundances as observed by the hydrobiological survey
Author: Gert-Jan Jeunen, Tatsiana Lipinskaya, Helen Gajduchenko, Viktoriya Golovenchik, Michail Moroz, Viktor Rizevsky, Vitaliy Semenchenko, Neil J. Gemmell
Data type: excel file
Explanation note: Macro-invertebrate abundances as observed by the hydrobiological survey. Scientific names are given in the respective column. Values indicate the number of individuals at a given sampling site. Sampling site notation follows the abbreviations of Suppl. material 1.
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Link: https://doi.org/10.3897/mbmg.6.68575.suppl6

Supplementary material 7
Rarefaction curves for each metabarcoding assay
Author: Gert-Jan Jeunen, Tatsiana Lipinskaya, Helen Gajduchenko, Viktoriya Golovenchik, Michail Moroz, Viktor Rizevsky, Vitaliy Semenchenko, Neil J. Gemmell
Data type: doxc file
Explanation note: Rarefaction curves for each metabarcoding assay (fish (16S); crustacean (16S)) per habitat for each sampling site. Number of taxa are indicated on the y-axis and number of reads on the x-axis. Sampling site notation follows the abbreviations of Suppl. material 1.
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Link: https://doi.org/10.3897/mbmg.6.68575.suppl7

Supplementary material 8

Environmental DNA detections from the passive surveillance for both metabarcoding assays

Author: Gert-Jan Jeunen, Tatsiana Lipinskaya, Helen Gajduchenko, Viktoriya Golovenchik, Michail Moroz, Viktor Rizevsky, Vitaliy Semenchenko, Neil J. Gemmell

Data type: excel file

Explanation note: Environmental DNA detections from the passive surveillance for both metabarcoding assays, i.e., fish (16S) and crustacean (16S). Scientific and common names are given in the respective columns. Values indicate the number of reads assigned to each taxonomic unit for a given sampling site. Sampling site notation follows the abbreviations of Suppl. material 1.

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